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A novel target to inhibit angiogenesis

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A novel target to inhibit angiogenesis

Field of the invention

The invention relates to the field of pathological angiogenesis. In particular the invention relates to the use of molecules binding to AC133 that can be used for the manufacture of a medicament to prevent angiogenesis.

Background of the invention

The "hemangioblast" is a putative progenitor cell that has the potential to form either endothelial or hematopoietic cells. It exists during embryogenesis in the blood island region of the yolk sac (1), which is therefore the earliest site of hematopoiesis and vasculogenesis. Until recently, vasculogenesis has been thought to be restricted to the yolk sac and the early embryogenesis. However, novel observations have revealed in adulthood a situation consistent with vasculogenesis: endothelial cells derived from angioblasts or "hemangioblasts" previously isolated from peripheral blood or bone marrow are incorporated into sites of neovascularization in physiological and pathological conditions (2-6). In addition, the number of these endothelial cell progenitors increases in the peripheral blood during tissue ischemia or following the administration of VEGF or GM-CSF, a cytokine known to induce mobilization of hematopoietic stem cells from the bone marrow into the peripheral blood (5, 6). Recent studies in humans, dogs, rats, rabbits and mice have indeed indicated the presence of endothelial precursor cells (EPCs) in bone marrow and peripheral blood during adult life which can be mobilized and incorporated into newly formed vessels or are involved in endothelialization of implants (4, 7-13). Interestingly, in all these experiments, EPCs are isolated together with other hematopoietic stem cells by using antibodies directed against hematopoietic stem cell antigens. AC133 is a rather novel human hematopoietic stem cell antigen (14) of unknown physiological or pathological function. AC133, recently designed CD133 (National Center for Biotechnology, 2000) is expressed on lineage non-committed stem and progenitor cells but not on mature peripheral blood cells and umbilical vein derived endothelial cells (15). It is detected on 30-60% of all $CD34^+$ cells, including $CD34^{bright}$ cells. This cell population contains $CD38^{dim/neg}$, $HLA\text{-}DR^-$, $CD117^+$, $CD90^+$ cells. $AC133^+$ $CD34^+$ hematopoietic stem cells

are enriched in Long-Term Culture-Initiating Cells, NOD/SCID repopulating cells and dendritic cell progenitors (16). Subsets of this population express the angiopoietin receptors TIE (67.6%) and TEK (36.8%), Flt-1 (7%), Flt-4 (3.2%), KDR (10.4%), the receptor tyrosine kinase HER-2 (15.4%) and Flt-3 (77.6%). Only few $AC133^+$ cells do not co-express CD34: these cells are very small and define a population of unknown delineation ($CD71^-$, $CD117^-$, $CD10^-$, $CD38^{low}$, $CD135^+$, $HLA\text{-}DR^{high}$) (17). In acute myeloid leukemias, AC133 expression is often but not always associated with CD34 expression (18, 19). AC133 is also found on acute lymphoid leukemia blasts and on a subset of $CD34^+$ B-cell precursors (20). Flow cytometric analyses of a wide panel of human cell lines showed that only retinoblastoma and teratocarcinoma cell lines express AC133 (21). Recently, AC133 was found to be expressed in EPCs. $CD34^+$ cells co-expressing VEGFR-2 and AC133 have been isolated from peripheral blood, cord blood, fetal liver and bone marrow. However, the possible role of AC 133 in hematopoiesis and vasculogenesis in the developing embryo and, after birth, in angiogenesis, postnatal vasculogenesis and hematopoietic stem cell trafficking, remains largely unknown. To study in detail the *in vivo* role of AC133 in the present invention AC133 deficient mice were generated. It was surprisingly found that AC 133 has a key role in pathological vasculogenesis and/or angiogenesis and that inhibitors of AC133 can be used in therapeutic strategies to inhibit blood vessel formation in various pathological disorders.

Aims and detailed description of the invention

AC133 is a protein of 97 KD with a 5-transmembrane structure (14). The 5-transmembrane structure appears at present restricted to the human AC133, the murine prominin and the related protein of the nematode C. elegans (25-28). This structure indicates that these proteins belong to a new class of serpentine receptors. Murine prominin has 60% amino acid homology with AC133 (25). It is not yet clear whether murine prominin is the murine homologue of human AC133, or whether it is a close family member (25, 26). For the sake of clarity the nucleotide sequence of human AC133 is designated here as SEQ ID NO: 1 and the amino acid sequence of human AC133 is designated as SEQ ID NO: 2. The present invention shows that inhibitors of AC133 can be used in therapeutic applications for the prevention of pathological angiogenesis.

Thus the invention provides in one embodiment the use of a molecule which comprises a region specifically binding to AC133 or nucleic acids encoding AC133, for the manufacture of a medicament to treat pathological angiogenesis.

According to the invention molecules that comprise a region specifically binding to AC133 or nucleic acids encoding AC133 which can be used for the manufacture of a medicament to treat pathological angiogenesis can be chosen from the list comprising an antibody or any fragment thereof binding to AC133, a (synthetic) peptide, a protein, a small molecule specifically binding to AC133 or nucleic acids encoding AC133, a ribozyme against nucleic acids encoding AC133, and anti-sense nucleic acids hybridising with nucleic acids encoding AC133.

The term 'antibody' or 'antibodies' relates to an antibody characterized as being specifically directed against AC133 or any functional derivative thereof, with said antibodies being preferably monoclonal antibodies; or an antigen-binding fragment thereof, of the $F(ab')_2$, F(ab) or single chain Fv type, or any type of recombinant antibody derived thereof. These antibodies of the invention, including specific polyclonal antisera prepared against AC133 or any functional derivative thereof, have no cross-reactivity to others proteins. The monoclonal antibodies of the invention can for instance be produced by any hybridoma liable to be formed according to classical methods from splenic cells of an animal, particularly of a mouse or rat immunized against AC133 or any functional derivative thereof, and of cells of a myeloma cell line, and to be selected by the ability of the hybridoma to produce the monoclonal antibodies recognizing AC133 or any functional derivative thereof which have been initially used for the immunization of the animals. The monoclonal antibodies according to this embodiment of the invention may be humanized versions of the mouse monoclonal antibodies made by means of recombinant DNA technology, departing from the mouse and/or human genomic DNA sequences coding for H and L chains or from cDNA clones coding for H and L chains. Alternatively the monoclonal antibodies according to this embodiment of the invention may be human monoclonal antibodies. Such human monoclonal antibodies are prepared, for instance, by means of human peripheral blood lymphocytes (PBL) repopulation of severe combined immune deficiency (SCID) mice as described in PCT/EP 99/03605 or by using transgenic non-human animals capable of producing human antibodies as described

in US patent 5,545,806. Also fragments derived from these monoclonal antibodies such as Fab, F(ab)'$_2$ and ssFv ("single chain variable fragment"), providing they have retained the original binding properties, form part of the present invention. Such fragments are commonly generated by, for instance, enzymatic digestion of the antibodies with papain, pepsin, or other proteases. It is well known to the person skilled in the art that monoclonal antibodies, or fragments thereof, can be modified for various uses. The antibodies involved in the invention can be labeled by an appropriate label of the enzymatic, fluorescent, or radioactive type.

In a specific embodiment the antibodies against AC133 can be derived from animals of the camelid family. In said family immunoglobulins devoid of light polypeptide chains are found. Heavy chain variable domain sequences derived from camelids are designated as VHH's. "Camelids" comprise old world camelids (*Camelus bactrianus* and *Camelus dromaderius*) and new world camelids (for example *Lama paccos*, *Lama glama* and *Lama vicugna*). EP0656946 describes the isolation and uses of camelid immunoglobulins and is incorporated herein by reference.

Small molecules, e.g. small organic molecules, and other drug candidates can be obtained, for example, from combinatorial and natural product libraries.

Also within the scope of the invention are oligoribonucleotide sequences, that include anti-sense RNA and DNA molecules and ribozymes that function to inhibit the translation of AC133 mRNA. Anti-sense RNA and DNA molecules act to directly block the translation of mRNA by binding to targeted mRNA and preventing protein translation. In regard to antisense DNA, oligodeoxyribonucleotides derived from the translation initiation site, e.g., between -10 and +10 regions of the AC133 nucleotide sequence, are preferred. Ribozymes are enzymatic RNA molecules capable of catalyzing the specific cleavage of RNA. The mechanism of ribozyme action involves sequence specific hybridization of the ribozyme molecule to complementary target RNA, followed by an endonucleolytic cleavage. Within the scope of the invention are engineered hammerhead motif ribozyme molecules that specifically and efficiently catalyze endonucleolytic cleavage of AC133 RNA sequences.

Specific ribozyme cleavage sites within any potential RNA target are initially identified by scanning the target molecule for ribozyme cleavage sites which include the following sequences, GUA, GUU and GUC. Once identified, short RNA sequences of

between 15 and 20 ribonucleotides corresponding to the region of the target gene containing the cleavage site may be evaluated for predicted structural features such as secondary structure that may render the oligonucleotide sequence unsuitable. The suitability of candidate targets may also be evaluated by testing their accessibility to hybridization with complementary oligonucleotides, using ribonuclease protection assays.

Both anti-sense RNA and DNA molecules and ribozymes of the invention may be prepared by any method known in the art for the synthesis of RNA molecules. These include techniques for chemically synthesizing oligodeoxyribonucleotides well known in the art such as for example solid phase phosphoramidite chemical synthesis. Alternatively, RNA molecules may be generated by *in vitro* and *in vivo* transcription of DNA sequences encoding the antisense RNA molecule. Such DNA sequences may be incorporated into a wide variety of vectors which incorporate suitable RNA polymerase promoters such as the T7 or SP6 polymerase promoters. Alternatively, antisense cDNA constructs that synthesize anti-sense RNA constitutively or inducibly, depending on the promoter used, can be introduced stably into cell lines.

The term 'pathological angiogenesis' refers to the formation and growth of blood vessels during the maintenance and the progression of several disease states. Examples where pathological angiogenesis can occur are blood vessels (atherosclerosis, hemangioma, hemangioendothelioma), bone and joints (rheumatoid arthritis, synovitis, bone and cartilage destruction, osteomyelitis, pannus growth, osteophyte formation, neoplasms and metastasis), skin (warts, pyogenic granulomas, hair growth, Kaposi's sarcoma, scar keloids, allergic oedema, neoplasms), liver, kidney, lung, ear and other epithelia (inflammatory and infectious processes (including hepatitis, glomerulonephritis, pneumonia), asthma, nasal polyps, otitis, transplantation, liver regeneration, neoplasms and metastasis), uterus, ovary and placenta (dysfunctional uterine bleeding (due to intrauterine contraceptive devices), follicular cyst formation, ovarian hyperstimulation syndrome, endometriosis, neoplasms), brain, nerves and eye (retinopathy of prematurity, diabetic retinopathy, choroidal and other intraocular disorders, leukomalacia, neoplasms and metastasis), heart and skeletal muscle due to work overload, adipose tissue (obesity), endocrine organs (thyroiditis, thyroid enlargement, pancreas transplantation), hematopoiesis (AIDS (Kaposi),

hematologic malignancies (leukemias, etc.), lymph vessels (tumour metastasis, lymphoproliferative disorders).

The term 'medicament to treat' relates to a composition comprising molecules as described above and a pharmaceutically acceptable carrier or excipient (both terms can be used interchangeably) to treat diseases as indicated above. Suitable carriers or excipients known to the skilled man are saline, Ringer's solution, dextrose solution, Hank's solution, fixed oils, ethyl oleate, 5% dextrose in saline, substances that enhance isotonicity and chemical stability, buffers and preservatives. Other suitable carriers include any carrier that does not itself induce the production of antibodies harmful to the individual receiving the composition such as proteins, polysaccharides, polylactic acids, polyglycolic acids, polymeric amino acids and amino acid copolymers. The 'medicament' may be administered by any suitable method within the knowledge of the skilled man. The preferred route of administration is parenterally. In parental administration, the medicament of this invention will be formulated in a unit dosage injectable form such as a solution, suspension or emulsion, in association with the pharmaceutically acceptable excipients as defined above. However, the dosage and mode of administration will depend on the individual. Generally, the medicament is administered so that the protein, polypeptide, peptide of the present invention is given at a dose between 1 µg/kg and 10 mg/kg, more preferably between 10 µg/kg and 5 mg/kg, most preferably between 0.1 and 2 mg/kg. Preferably, it is given as a bolus dose. Continuous infusion may also be used and includes continuous subcutaneous delivery via an osmotic minipump. If so, the medicament may be infused at a dose between 5 and 20 µg/kg/minute, more preferably between 7 and 15 µg/kg/minute.

In another embodiment antibodies or functional fragments thereof can be used for the manufacture of a medicament for the treatment of the above mentioned disorders. As a non-limiting example there are the antibodies described in US 5,843,633. In a specific embodiment said antibodies are humanized (Rader et al., 2000, J. Biol. Chem. 275, 13668) and more specifically human antibodies are used to manufacture a medicament to treat pathological angiogenesis. In yet another specific embodiment antibodies derived from camelids are used to manufacture a medicament to treat pathological angiogenesis.

Another aspect of administration for treatment is the use of gene therapy to deliver the above mentioned anti-sense gene or functional parts of the AC133 gene or a ribozyme directed against the AC133 mRNA or a functional part thereof. Gene therapy means the treatment by the delivery of therapeutic nucleic acids to patient's cells. This is extensively reviewed in Lever and Goodfellow 1995; Br. Med Bull.,51, 1-242; Culver 1995; Ledley, F.D. 1995. Hum. Gene Ther. 6, 1129. To achieve gene therapy there must be a method of delivering genes to the patient's cells and additional methods to ensure the effective production of any therapeutic genes. There are two general approaches to achieve gene delivery; these are non-viral delivery and virus-mediated gene delivery.

The invention also provides methods for identifying compounds or molecules which bind on AC133 and prevent or suppress pathological angiogenesis. With "suppression" it is understood that said suppression can occur for at least 20%, 30%, 30%, 50%, 60%, 70%, 80%, 90% or even 100%.

Thus in another embodiment the invention provides a method to identify molecules that comprise a region that specifically binds to AC133 comprising: (1) exposing AC133 or nucleic acids encoding AC133 to at least one molecule whose ability to suppress or prevent pathological angiogenesis is sought to be determined, (2) determining binding or hybridising of said molecule(s) to AC133 or nucleic acids encoding AC133, and (3) monitoring said pathological angiogenesis when administering said molecules as a medicament.

The latter method is also referred to as 'drug screening assay' or 'bioassay' and typically include the step of screening a candidate/test compound or agent for the ability to interact with AC133. Candidate compounds or agents, which have this ability, can be used as drugs to combat or prevent pathological conditions of angiogenesis. Candidate/test compounds such as small molecules, e.g. small organic molecules, and other drug candidates can be obtained, for example, from combinatorial and natural product libraries as described above.

Typically, the assays are cell-free assays which include the steps of combining AC133 and a candidate/test compound, e.g., under conditions which allow for interaction of (e.g. binding of) the candidate/test compound with AC133 to form a complex, and detecting the formation of a complex, in which the ability of the candidate compound to

interact with AC133 is indicated by the presence of the candidate compound in the complex. Formation of complexes between AC133 and the candidate compound can be quantitated, for example, using standard immunoassays. The AC133 employed in such a test may be free in solution, affixed to a solid support, borne on a cell surface, or located extracellularly or even intracellularly.

To perform the above described drug screening assays, it is feasible to immobilize AC133 or its (their) target molecule(s) to facilitate separation of complexes from uncomplexed forms of one or both of the proteins, as well as to accommodate automation of the assay. Interaction (e.g., binding of) of AC133 to a target molecule, can be accomplished in any vessel suitable for containing the reactants. Examples of such vessels include microtitre plates, test tubes, and microcentrifuge tubes. In one embodiment, a fusion protein can be provided which adds a domain that allows the protein to be bound to a matrix. For example, AC133-His tagged can be adsorbed onto Ni-NTA microtitre plates, or AC133-ProtA fusions adsorbed to IgG, which are then combined with the cell lysates (e.g., ^{35}S-labeled) and the candidate compound, and the mixture incubated under conditions conducive to complex formation (e.g., at physiological conditions for salt and pH). Following incubation, the plates are washed to remove any unbound label, and the matrix immobilized and radiolabel determined directly, or in the supernatant after the complexes are dissociated. Alternatively, the complexes can be dissociated from the matrix, separated by SDS-PAGE, and the level of AC133-binding protein found in the bead fraction quantitated from the gel using standard electrophoretic techniques. Other techniques for immobilizing protein on matrices can also be used in the drug screening assays of the invention. For example, AC133 can be immobilized utilizing conjugation of biotin and streptavidin. Biotinylated AC133 can be prepared from biotin-NHS (N-hydroxy-succinimide) using techniques well known in the art (e.g., biotinylation kit, Pierce Chemicals, Rockford, Ill.), and immobilized in the wells of streptavidin-coated 96 well plates (Pierce Chemical). Another technique for drug screening which provides for high throughput screening of compounds having suitable binding affinity to AC133 is described in detail in "Determination of Amino Acid Sequence Antigenicity" by Geysen HN, WO 84/03564, published on 13/09/84. In summary, large numbers of different small peptide test compounds are synthesized on a solid substrate, such as plastic pins or some other surface. The protein test compounds are reacted with fragments of AC133 and washed. Bound AC133 is then detected by methods well known in the art. Purified

AC133 can also be coated directly onto plates for use in the aforementioned drug screening techniques. Alternatively, non-neutralizing antibodies can be used to capture the peptide and immobilize it on a solid support. This invention also contemplates the use of competitive drug screening assays in which neutralizing antibodies capable of binding AC133 specifically compete with a test compound for binding AC133. In this manner, the antibodies can be used to detect the presence of any protein, which shares one or more antigenic determinants with AC133.

Examples

1. Generation of a AC133 knock-out mouse

Targeted inactivation of the *AC133* gene was achieved by deletion of exon 2 (containing the start codon). Briefly, a genomic BAC (bacterial artificial chromosome) containing the murine *AC133* (14) was obtained from Research Genetics Inc (Huntsville, AL) after screening by PCR and hybridization. Mapping of the murine *AC133* homologue gene revealed that the first exon, which is 79 bp long, is separated from the second exon by an approximately 8 kb intron. It is the second exon (376 bp long) which contains the startcodon ATG. A BamHI fragment of 11.5 kb containing exon 2 was subcloned into the pUC18 plasmid. A targeting vector for inactivation of the *AC133* gene, p*PNT.AC133*^{null}, was constructed consisting of, from 5' to 3': 1.2 kb of 5' homology comprising the end of intron 1; a *lox*P-flanked *neomycin* gene; 5.5 kb from intron 2 as 3'-homology; and a *thymidine kinase* selection cassette outside of the regions of homology for counterselection against random integration events. The integrity of the construct was verified by restriction digestion and sequencing. The linearized targeting vector p*PNT.AC133*^{null} was electroporated in R1 ES cells and targeted clones were identifed by appropriate Southern blot analysis and used for morula aggregation to generate AC133 deficient chimeric and germline mice. AC133 $^{-/-}$ mice were born at the expected Mendelian frequency (~25% of 450 offspring from AC133 $^{+/-}$ breeding pairs). They appeared healthy and were fertile, irrespective of their genetic background (backgrounds tested: 50% Swiss/50% 129, 100% 129, 50% C57Bl6/50% 129 and 50% NMRI/50% 129). We anticipated that AC133 might play a crucial role in hematopoiesis implying that the AC133$^{-/-}$ embryo would die *in utero* either after the appearence of the primitive hematopoiesis (7.5 days post coitum, site:

yolk sac) or at the emergence of the definitive hematopoiesis (12.5 days post coitum, site: fetal liver). Surprisingly, however, embryonic development in AC133$^{-/-}$ mice was normal. AC133$^{-/-}$ embryos were not rescued by maternal AC133, as AC133$^{-/-}$ embryos, sired by AC133$^{+/-}$ as well as by AC133$^{-/-}$ breeding pairs, developed normally. Also postnatal physiological vascular development seemed normal since no vascular defects could be observed in the heart, kidneys, lungs and skeletal muscle during postnatal growth in AC133$^{-/-}$ mice.

2. Impaired pathological angiogenesis in AC133 knock-out mice

In order to study the possible role of AC133 in pathological conditions of angiogenesis AC 133$^{-/-}$ mice and their wild-type littermates were subjected to a mouse model of ischemic retinopathy. In this hyperoxia-induced retinopathy model, neonatal mice (with an immature retinal vasculature) are exposed to hyperoxia, resulting in obliteration of the developing blood vessels supplying oxygen to the retina. When the mice are then returned to normoxia, the retina, distal to the occluded vessels, becomes ischemic, inducing VEGF production and ultimately resulting in reproducible and quantifiable proliferative retinal neovascularization (29, 30). This model, which mimicks to a certain extent the vascular response during retinopathy of prematurity or diabetic retinopathy, may be useful to test the efficacy of (anti)-angiogenic molecules (31). Mouse pups of 7 days (P7) together with their mothers, are subjected to hyperoxia (75% oxygen) in specially designed oxygen chambers for 5 days, without opening the cages. On P12, the animals are returned to room air until P17, when the retinas are assessed for maximal neovascular response. Anesthetized mice are perfused through the left ventricle with 1 ml of phosphate buffered saline containing 50 mg of 2x10^6 molecular weight fluorescein-dextran. The eyes are removed and fixed in 4% paraformaldehyde for 3 (right eye) or 24 (left eye) hrs. Of the right eyes, lenses are removed and peripheral retinas cut to allow flat mounting with glycerol-gelatin. The flat mounted retinas are analyzed by fluorescence microscopy. The left eyes are embedded in paraffin and serial 6 µm sections are cut sagittally throughout the cornea, parallel to the optic nerve, and stained with hematoxylin-eosin. The proliferative neovascular response is quantified by counting the number of new vessels (= tufts) and the number of endothelial cells extending from the internal limiting membrane of the retina into the vitreum on the stained sagittal cross-sections. The angiographic technique using

fluorescein-dextran perfusion is used in conjunction with this counting method for rapid screening of retinas or as an alternative grading system for quantitative evaluation.

Loss of AC133 significantly protected mice against intra-vitreous neovascularization, as evaluated by counting the number of neovascular tufts and endothelial cells (EC) in the vitrous cavity (n=15; $p<0.001$)

	N° of tufts in vitreous cavity (per 10 retinal sections)	N° of EC in vitreous cavity (per 10 retinal sections)
$AC133^{+/+}$ pups (n = 15)	157.1 ± 13.6	286.0 ± 45.1
$AC133^{-/-}$ pups (n = 15)	72.5 ± 14.6	106.2 ± 22.6

The effect of (anti)-angiogenic molecules has been frequently studied in the corneal micropocket assay [Kenyon, 1996]. Implantation of hydron pellets containing angiogenic factors in the stroma of the avascular mouse cornea allows screening of the angiogenic response in the absence of inflammation. As the cornea in the mouse is thinner than in other species, growth of new blood vessels largely occurs in a two-dimensional plane. Hydron pellets containing an angiogenic substance (like bFGF or VEGF) are implanted into the corneal stroma adjacent to the temporal limbus. This induces neovascularization of the avascular corneal stroma from day 3 to day 8 after implantation, without substantial corneal edema or inflammation. Like the retinal hypoxia model, it gives a predictable, persistent and aggressive neovascular response, which is dependent on direct stimulation of blood vessels rather than on indirect stimulation by the induction of inflammation [Kenyon, 1996]. The mouse corneal micropocket assay was performed as previously described [Asahara, 1998]. Hydron-coated sucralfate pellets containing 300 ng of $VEGF_{165}$ were positioned 1 mm from the corneal limbus. Corneal angiogenesis was evaluated six days after implantation of hydron pellets. Anesthetized mice were perfused through the left ventricle with 1 ml of phosphate buffered saline containing 50 mg of $2x10^6$ molecular weight fluorescein-dextran. The eyes were removed and fixed in 4% paraformaldehyde for 3 hrs. Corneas were pealed of and flat mounted with glycerol-gelatin. Vessel length, the arc of circumference occupied by neovascularity

(circumferential neovascularity) and the integrated optical density of the area occupied by new vessels was determined by fluorescence microscopy and image analysis.

Mice deficient for AC133 showed a reduced angiogenic response. Despite a comparable length of the newly formed vessels, the circumferential neovascularity and the integrated optical density of the vessel area were significantly lower in the AC133 mice (n=7; $p<0.05$).

	Vessel length (μm)	Circumferential neovascularity (μm)	Integrated optical density of the vessel area
$AC133^{+/+}$ pups (n = 7)	5.3 ± 0.6	41.7 ± 3.0	430 ± 58
$AC133^{-/-}$ pups (n = 7)	5.1 ± 0.7	28.9 ± 3.5 *	302 ± 37 *

Vascular remodeling was also studied in a model of skin wound healing as described before [Frank, 1995; Carmeliet, 2001]. For skin wounding, a standardised 15 mm full-thickness skin incision was made on the back of the mice, taking care not to damage the underlying muscle. Wound healing was quantified by daily measurements of the width and the length of the wound. New blood vessel formation was analysed on skin sections harvested four days after wounding. Using antibodies to CD31, which labels endothelial cells, and antibodies to smooth muscle alpha actin, labeling smooth muscle cells, the number of new vessels and of new vessels covered with SMC were analysed. Both genotypes contained comparable densities of vessels in unwounded skin. However, the number of capillaries infiltrating the wound as well as the number of smooth muscle-coated vessels in the wounded area were significantly reduced in AC133 deficient mice (n=5; $p<0.05$).

	Capillary density in wounded area (/ mm^2)	Density of SMC coated vessels (/ mm^2)
$AC133^{+/+}$ pups (n = 5)	±	53.4 ± 5.9
$AC133^{-/-}$ pups (n = 5)	±	28.6 ± 2.7 *

The role of PROM-1 in pathological vasculogenesis and angiogenesis

General introduction

Prominin-1 (PROM-1), also called AC133 or CD133, is a rather novel human hematopoietic stem cell antigen [1] of unknown physiological or pathological function. AC133-antigen was first detected on $CD34^{bright}$ hematopoietic stem cells [2] and has since been widely used to facilitate the analysis and isolation of hematopoietic and primitive cells [3-5]. Only few $AC133^+$ cells do not coexpress CD34: these cells are very small and define a population of unknown delineation [6]. In acute myeloid leukemias, PROM-1 expression is often but not always associated with CD34 expression [7,8]. AC133is also found on acute lymphoid leukemia blasts and on a subset of $CD34^+$ B-cell precursors [9]. Flow cytometry analyses of a wide panel of human cell lines showed that only retinoblastoma and teratocarcinoma cell lines express AC133[10]. More recently, it was shown that endothelial progenitor cells co-express PROM-1 antigen and the endothelial cell-specific receptor kinase-inert domain-containing acceptor (KDR) in subpopulations of $CD34^+$ cells derived from fetal liver, bone marrow, cord blood and peripheral blood [11,12]. Recently, human central nervous system stem cells were also reported to express AC133-antigen [13]. A characteristic feature of this protein is its rapid down-regulation during cell differentiation [12,14], which makes it a unique cell surface marker for the identification and isolation of stem cells an,d progenitor cells.

Human PROM-1 antigen is a glycoprotein of 120 KD and contains an extracellular N terminus, two extracellular loops, five transmembrane domains, tow small cysteine-rich cytoplasmic loops and a cytoplasmic C terminus [1]. Recently a novel isoform of human PROM-1 with a 27 basepair deletion has been described [15]. A structural and sequence-related protein, was identified as the mouse orthologue of human PROM-1 [14]. The 5-transmembrane structure appears phylogenetically conserved from mammals to zebrafish and in fruit flies and nematodes [16,17]. Murine prominin, which has a 65% amino acid homology with human PROM-1 also exists in two isoforms. The short human and murine prominin isoform both encode proteins that lack a 9-amino acid segment at the same location in the N-terminal extracellular region just proximal to the first transmembrane domain [15,18,19].

Although human PROM[redacted]has been used as a cell surface m[redacted]r to identify and isolate certain stem cell and progenitor cell populations, the molecular mechanism of how this protein functions remain unclear. To study the in vivo role of PROM-1 in hematopoiesis, vasculogenesis, angiogenesis and possibly neurogenesis, we generated PROM-1 deficient mice.

The role of PROM-1 in blood vessel formation

Background and significance

The "hemangioblast" is a putative progenitor cell that has the potential to form either endothelial or hematopoietic cells. It exists during embryogenesis in the blood island region of the yolk sac [20], which is therefore the earliest site of hematopoiesis and vasculogenesis. Until recently, vasculogenesis has been thought to be restricted to the yolk sac and the early embryogenesis. However, novel observations have revealed in adulthood a situation consistent with vasculogenesis: endothelial cells derived from angioblasts or "hemangioblasts" previously isolated from peripheral blood or bone marrow are incorporated into sites of neovascularization in physiological and pathological conditions [21-25]. In addition, the number of these endothelial cell progenitors increases in the peripheral blood during tissue ischemia or following the administration of VEGF or GM-CSF, a cytokine known to induce mobilization of hematopoietic stem cells from the bone marrow into the peripheral blood [24,25]. Recent studies in humans, dogs, rats, rabbits and mice have indeed indicated the presence of endothelial precursor cells (EPCs) in bone marrow and peripheral blood during adult life which can be mobilized and incorporated into newly formed vessels or are involved in endothelialization of implants [23,26-32].

Interestingly, in all these experiments, endothelial cell progenitors are isolated together with other hematopoietic stem cells by using antibodies directed against hematopoietic stem cell antigens. PROM-1 is expressed on lineage non-committed stem and progenitor cells but not on mature peripheral blood cells and umbilical vein derived endothelial cells [2]. $CD34^+$ cells co-expressing VEGFR-2 and PROM-1, recently designed CD133 (National Center for Biotechnology, 2000) have been isolated from peripheral blood, cord blood, fetal liver and bone marrow. When grown in the presence of VEGF and FGF-2 or the novel cytokine stem cell growth factor

(SCGF), these cells give rise to endothelial cells, thus suggesting that this subset of $CD34^+$, $VEGFR\text{-}2^+$ & $CD133^+$ cells may play a role in neovasculogenesis [3,5,11].

Long term objectives

1. To study the possible role of PROM-1 in hematopoiesis and vasculogenesis in the developing embryo.

2. To determine a possible role for PROM-1 in postnatal vasculogenesis, angiogenesis and hematopoietic stem cell trafficking, in pathological conditions of blood vessel formation.

Preliminary results

- To study the *in vivo* role of PROM-1, PROM-1 (prominin) deficient mice were generated. Targeted inactivation of the PROM-1 gene was achieved by deletion of exon 2 (containing the start codon). Briefly, a genomic BAC (bacterial artificial chromosome) containing the murine PROM-1 [1] was obtained from Research Genetics Inc (Huntsville, AL) after screening by PCR and hybridization. Mapping of the murine PROM-1 homologue gene revealed that the first exon, which is 79 bp long, is separated from the second exon by an approximately 8 kb intron. It is the second exon (376 bp long) that contains the startcodon ATG. A BamHI fragment of 11.5 kb containing exon 2 was subcloned into the pUC18 plasmid. A targeting vector for inactivation of the PROM-1 gene, p*PNT*.PROM-1^{null}, was constructed consisting of, from 5' to 3': 1.2 kb of 5' homology comprising the end of intron 1; a *lox*P-flanked *neomycin* gene; 5.5 kb from intron 2 as 3'-homology; and a *thymidine kinase* selection cassette outside of the regions of homology for counterselection against random integration events. The integrity of the construct was verified by restriction digestion and sequencing. The linearized targeting vector p*PNT*.PROM-1^{null} was electroporated in R1 ES cells and targeted clones were identifed by appropriate Southern blot analysis and used for morula aggregation to generate PROM-1 deficient chimeric and germline mice.

- PROM-1 deficient mice were born at the expected Mendelian frequency (~25% of 450 offspring from PROM-1 heterozygous breeding pairs). They appeared healthy and were fertile, irrespective of their genetic background (backgrounds tested: 50% Swiss/50% 129, 100% 129, 50% C57Bl6/50% 129). We anticipated that PROM-1 might play a crucial role in hematopoiesis implying that the PROM-$1^{-/-}$ embryo would die *in utero* either after the appearence of the

primitive hematopoiesis (7.5 days post coitum, site: yolk sac) at the emergence of the definitive hematopoiesis (12.5 days post coitum, site: fetal liver). Surprisingly, however, embryonic development in PROM-1$^{-/-}$ mice was normal. PROM-1$^{-/-}$ embryos were not rescued by maternal PROM-1, as PROM-1$^{-/-}$ embryos, sired by PROM-1$^{+/-}$ as well as by PROM-1$^{-/-}$ breeding pairs, developed normally. Also postnatal physiological vascular development seemed normal since no vascular defects could be observed in the heart (capillary density is 5810 ± 154 in PROM-1$^{+/+}$ pups versus 5394 ± 179 in PROM-1$^{-/-}$ pups, n=3; p=NS), kidneys (number of glomeruli), lungs and skeletal muscle during postnatal growth in PROM-1$^{-/-}$ mice.

➢ In order to study the possible role of PROM-1 in pathological conditions of angiogenesis AC 133$^{-/-}$ mice and their wild-type littermates are or will be subjected to various murine models of pathological blood vessel formation.

Hyperoxia-induced retinopathy model: Neonatal mouse pups of 7 days (P7) (with an immature retinal vasculature) are exposed to hyperoxia (75% oxygen) for 5 days, resulting in obliteration of the developing blood vessels supplying oxygen to the retina. When the mice are then returned to normoxia, the retina becomes ischemic, inducing VEGF production and ultimately resulting in reproducible and quantifiable proliferative neovascularization in the vitreous cavity [33,34]. Preliminary results showed that loss of PROM-1 significantly protected mice against intravitreous neovascularization, as evaluated by counting the number of neovascular tufts in the vitrous cavity (157.1 ± 13.6 in PROM-1$^{+/+}$ pups versus 72.5 ± 14.6 in PROM-1$^{-/-}$ pups, n=15; p<0.001).

However, since it has been published recently, that a mutation in PROM-1 in human causes retinal degeneration possibly because of impaired regeneration of the evaginations and/or impaired conversions of the evaginations to disks [16], we started to look for retinal degeneration in our PROM-1 deficient mice. Retinal degeneration was indeed observed at 100% incidence in PROM-1 deficient mice even at young age (P21) (n= 4 for P21 and n= 3 for adult mice). Semithin sections clearly revealed that the photoreceptor layer, containing the rods and cones, and the outer nuclear layer are significantly reduced. Immunostainings in the retina showed that GFAP (astrocytes & Muller cells) and vimentin (Muller cells) expression were significantly upregulated in the PROM-1 deficient mice.

Less NSE and rhodopsin immunopositive photoreceptor cells were found in the photoreceptor and in the outer nuclear layer, while the number of CD90 positive ganglion cells in the ganglion cell layer, the number of syntaxin-1 amacrine cells in the inner plexiform layer and the number of PKC positive bipolar cells in the outer plexiform layer were identical in the two genotypes.

Corneal micropocket assay: Hydron pellets containing an angiogenic substance (like bFGF or VEGF) are implanted into the corneal stroma adjacent to the temporal limbus. This induces neovascularization of the avascular corneal stroma from day 3 to day 8 after implantation, without substantial corneal edema or inflammation. Like the retinal hypoxia model, it gives a predictable, persistent and aggressive neovascular response, which is dependent on direct stimulation of blood vessels rather than on indirect stimulation by the induction of inflammation [35]. The mouse corneal micropocket assay was performed as previously described [36]. Mice deficient for PROM-1 showed a reduced angiogenic response. The lenght of the newly formed vessels (0.93 ± 0.12 mm in PROM-$1^{+/+}$ mice versus 0.70 ± 0.03 mm in PROM-$1^{-/-}$ mice, n=6; $p<0.005$) as well as the circumferential neovascularity (6.23 ± 0.55 mm in PROM-$1^{+/+}$ mice versus 3.60 ± 0.32 mm in PROM-$1^{-/-}$ mice, n=6; $p<0.005$) and the integrated optical density of the vessel area (497 ± 100 in PROM-$1^{+/+}$ mice versus 196 ± 27 in PROM-$1^{-/-}$ mice, n=6; $p<0.05$) were significantly lower in the PROM-1 mice. Moreover, WT bone marrow transplantation into PROM-1 deficient mice rescued the impaired angiogenic response. The lenght of the newly formed vessels (0.57 ± 0.03 mm in PROM-$1^{+/+}$ mice versus 0.57 ± 0.03 mm in PROM-$1^{-/-}$ mice, n=6; $p<0.005$) as well as the circumferential neovascularity (3.52 ± 0.26 mm in PROM-$1^{+/+}$ mice versus 3.29 ± 0.22 mm in PROM-$1^{-/-}$ mice, n=6; p=NS) were identical in the both PROM-1 deficient and WT mice after WT bone marrow transplantation.

Model of skin wound healing: Vascular remodeling was also studied in a model of skin wound healing as described before [37,38]. For skin wounding, a standardised 15 mm full-thickness skin incision was made on the back of the mice, taking care not to damage the underlying muscle. Wound healing was quantified by daily measuring the width and the length of the wound. New blood vessel formation was analysed on skin sections harvested four days after wounding.

Wound healing was significantly impaired in the PROM-1 deficient mice as shown by the figure below. Both genotypes contained comparable densities of vessels in unwounded skin. However, the number of capillaries infiltrating the wound (185.8 ± 11.1 vessels/mm^2 in PROM-1$^{+/+}$ mice versus 135.0± 12.7 in PROM-1$^{-/-}$ mice, n=5; p<0.05), as well as the number of smooth muscle-coated vessels in the wounded area (58.2 ± 10 vessels/mm^2 in PROM-1$^{+/+}$ mice versus 28.6 ± 4.022 in PROM-1$^{-/-}$ mice, n=5; p<0.05) were significantly reduced in PROM-1 deficient mice.

Matrigel assay: Ingrowth of capillaries was also studied in a matrigel assay performed as described [39]. The angiogenic response in the matrigel of PROM-1$^{-/-}$ mice seemed somewhat lower as measured by the hemoglobin content per matrigel implant (137.0 ± 20.4 µg/ml in PROM-1$^{+/+}$ mice *versus* 112.1 ± 17.6 µg/ml in PROM-1$^{-/-}$ mice; n=15; p= NS). Histological sections of matrigel were then analysed for infiltration of leukocytes and for blood vessel formation after staining for inflammatory cells (CD45) and endothelial cells (CD31), respectively. The number of infiltrating leukocytes did not seem to differ but a reduced blood vessel formation (CD31 positive endothelial cells) was noticed in the matrigel implanted in PROM-1 deficient animals (% of CD31 positive area in matrigel: 0.55 ± 0.08% in PROM-1$^{+/+}$ mice *versus* 0.26 ± 0.06% in PROM-1$^{-/-}$ mice; n=5; p<0.05).

Myocardial infarction model: Myocardial infarction was performed by ligation of the LAD as described [40]. After 4 to 7 days, infarcted hearts were used for histological analysis or for immunostaining of thrombomodulin (endothelial cells) or smooth muscle alpha-actin (smooth muscle cells) [38]. Morphometric analysis and counting of immunoreactive cells was performed using a Quantimet Q600 image analysis system (Leica, Brussels, Belgium).

No differences were observed in the number of capillaries at 4 (490.9 ± 65.4 vessels/mm^2 in PROM-1$^{+/+}$ mice *versus* 493.3 ± 87.7 vessels/mm^2 in PROM-1$^{-/-}$ mice; n=3; p= NS) or 7 days (510.6 ± 28.3 vessels/mm^2 in PROM-1$^{+/+}$ mice *versus* 507.8 ± 24.6 vessels/mm^2 in PROM-1$^{-/-}$ mice; n=10; p= NS) after ligation or in the number of SMC covered vessels at 4 (20.3 ± 3.2 vessels/mm^2 in PROM-1$^{+/+}$ mice *versus* 23.3 ± 6.73 vessels/mm^2 in PROM-1$^{-/-}$ mice; n=3; p= NS) or at 7

days (87.6 ± 14.3 vessels/mm^2 in PROM-1$^{+/+}$ mice *versus* 76.4 ± 12.2 vessels/mm^2 in PROM-1$^{-/-}$ mice; n=10; p= NS) in the infarted area of hearts of PROM-1 deficient mice and wild-type littermates. However, a clear significant difference was observed in the number of infiltrating macrophages at 7 days after ligation (% of Mac3 positive area: 3.75 ± 0.77 % in PROM-1$^{+/+}$ mice *versus* 1.62 ± 0.42 % in PROM-1$^{-/-}$ mice; n=10; p< 0.05).

Hind limb ischemia model: Hind limb ischemia will be induced as described [41]. Unilateral right or bilateral ligations of the femoral artery and vein (proximal to the popliteal artery) and the cutaneous vessels branching from the caudal femoral artery side branch will be performed and two superficial preexisting collateral arterioles, connecting the femoral and saphenous artery, will be used for analysis. Genetic consequences on post-ischemic revascularization will be determined 14 days after ligation, using vascular morphological (histological evaluation of capillary density and SMC-coated vessel density, histological evaluation of myocyte necrosis and regeneration), perfusional (fluorescent microspheres, laser Doppler imaging), and functional (graded treadmill exercise or swim endurance excercise) analyses.

In a first experiment, no differences were found in the number or size of the main collateral or its second and third side branches in the adductor muscles of PROM-1 deficient and wild-type animals. We could not observe any genotypic differences in blood flow, measured by using fluorescent microspheres or with laser doppler imaging. Also, the myocyte necrosis and muscle regeneration in the ischemic gastrocnemius muscle was identical in both PROM-1 deficient mice and their wild-type littermates.

Tumor models

The role of PROM-1 will also be tested in tumor models. The following mouse models are operational and will be used to analyze tumor angiogenesis in vivo: 1) subcutaneous injection of ras-transformed fibroblasts in athymic nude (nu/nu) mice, 2) subcutaneous injection of Lewis lung carcinoma cells in syngenic C57Bl6 hosts, and 3) subcutaneous inoculation of rat C6 glioma cells of athymic nude (nu/nu) mice. Five to twenty million of tumor cells will be inoculated in the mice and tumor growth will be followed up to 30 days. Tumors will be measured with calipers and tumor volumes calculated using the formula [π/6 x (w1 x w2 x w2)],

where "w1" and "w2" represent the largest and smallest tumor diameter, respectively. Tumor vessel density and size will be determined on tissue sections using immunohistochemistry for visualization of endothelial cells (CD-31), in combination with quantitative morphometry of vascular densities and patterning. If necessary, intratumor flow will be determined using colored microspheres to quantitate flow across the entire tumor.

RAS transformed fibroblasts of PROM-1 deficient and WT mice were injected subcutaneously in athymic nude WT mice. Tumor growth was significantly reduced after inoculation of PROM-1 deficient tumor cells (tumor weight after 14 days: 1.1 ± 0.3 g in PROM-$1^{+/+}$ derived tumors versus 0.5 ± 0.2 g in PROM-$1^{-/-}$ derived tumors; n=7; $p< 0.05$). Histological analysis of the tumor sections revealed that the vessel density was reduced in the PROM-1 deficient tumors (513.2 ± 48.8 vessels/mm^2 in PROM-$1^{+/+}$ mice versus 396.3 ± 33.9 vessels/mm^2 in PROM-$1^{-/-}$ mice; n=7; p= NS). No difference in mean vessel area was observed. However, when WT RAS transformed fibroblasts were injected in PROM-1 deficient and WT nude mice, no difference in tumor weight was seen (tumor weight after 14 days: 0.9 ± 0.1 g in PROM-$1^{+/+}$ hosts versus 1.1 ± 0.3 g in PROM-$1^{-/-}$ hosts; n=7; $p< 0.05$). Blood vessel analysis is being performed.

Inoculation of LLC tumor cells (20.10^6 cells) in PROM-1 deficient and WT mice (50% Bl6/50%129) induced tumors after 2 days. Tumor weight was reduced in the PROM-1 deficient hosts during the first days after inoculation but seemed to catch up after 1 week (see table). Vessel growth was analysed in tumors harvested 14 days after inoculation. No differences were found in vessel density or in vessel area. In a second experiment tumors were harvested after 4 days and they are now being analysed.

Table: Weight of LLC tumors

Trial 1	WT		HO			
	Average	SEM	Average	SEM	n	Bonferroni
Day2	0.79	0.077	0.5	0.044	5	
Day4	0.85	0	0.55	0.037	5	
Day6	1.02	51	0.867	0.058	5	
Day8	1.31	0.085	1.128	0.086	5	
Day10	1.75	0.11	1.71	0.11	5	p=0.024
Day12	1.9	0.14	1.89	0.16	5	NS
Day14	2.22	0.13	2.49	0.16	5	NS

Trial 2	WT		HO			
	Average	SE	Average	SE	n	**Bonferroni**
Day2	0.089	0.064	0.56	0.04	8	
Day4	1.02	0.058	0.63	0.06	8	p=0.0002

LPS induced venous thrombosis in footpad

To study whether PROM-1 is important in inflammatory processes, a chronic inflammation foodpad assay was used. Endotoxin (20µl, E. Coli lipopolysaccharide, 5 and 50 µg/ml) was injected into the right footpad of both PROM-1 deficient and WT mice as described (Carmeliet et al, PAI II). Saline will be injected into the left footpad as a control. After 5 days, mice were sacrificed and both right and left footpad were measured with callipers, excised and fixed in 1% paraformaldehyde for 24 hours. Subsequently, footpads were embedded in paraffin and sectioned. Veins will be scored on haematoxilin and eosin stained sections for the presence of thrombi.

Five days after injecting 50 µg/ml of endotoxin, a decrease in footpad thickness was observed in the PROM-1 deficient compared to their WT controls.

- To study the role of PROM-1 in the involvement of endothelial progenitor cells (EPCs), murine splenocytes of PROM-1 deficient mice and their wild-type littermates were isolated and subjected to an *in vitro* endothelial progenitor cell

assay (EPC) assay[redacted]. Splenocytes were isolated by F[redacted] fractionation of murine spleen and plated on fibronectin-coated 24-well plates. After 4 days of culture, cells were stained with DiI-labeled acetyl-LDL and Alexa 488-conjugated isolectin-B4 and examined with a confocal microscope. EPCs are identified as acetyl-LDL and isolectin-B4 double-positive cells. Initial experiments revealed an increased number of EPCs in cultures obtained from wild-type spleens compared to those from AC deficient spleens (358 ± 41/ mm^2 in PROM-$1^{+/+}$ mice versus 179 ± 19/ mm^2 in PROM-$1^{-/-}$ mice, n=3; p=0.0171).

- To investigate the role of PROM-1 in EPC mobilization from bone marrow in conditions of pathological blood vessel formation, EPC assays using cells from mice subjected to the hindlimb ischemia model (see above) will be performed. The first time point will be 7 days post-ligation, since after hindlimb ischemia maximal EPC mobilization was shown at this time point [23].
- RNA of PROM-1 deficient and WT EPCs will be used to analyze expression of VEGF, PlGF, Flk1, Flt1, etc. by RT-PCR.
- In order to study the in vivo role of PROM-1 in the involvement of hematopoietic progenitors or vascular progenitors in blood vessel formation, PROM-1 deficient and wild-type mice will be subjected to wild-type bone marrow transplantations or transplantations of population enriched progenitors. Four weeks after sublethal irradiation and transplantation, mice will be subjected to some of the angiogenesis assays described above. (see corneal micropocket)

All these preliminary data clearly indicate a role of AC 133 in pathological vasculogenesis and/or angiogenesis and implicate the use of inhibitors of PROM-1 in therapeutic strategies to inhibit blood vessel formation in various pathological disorders.

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Claims

1. Use of a molecule which comprises a region specifically binding to AC133 or nucleic acids encoding AC133, for the manufacture of a medicament to treat pathological angiogenesis.

2. Use according to claim 1 wherein said molecule is chosen from the list comprising an antibody or any fragment thereof binding to AC133, a small molecule specifically binding to AC133 or nucleic acids encoding AC133, a ribozyme against nucleic acids encoding AC133, and anti-sense nucleic acids hybridising with nucleic acids encoding AC133.

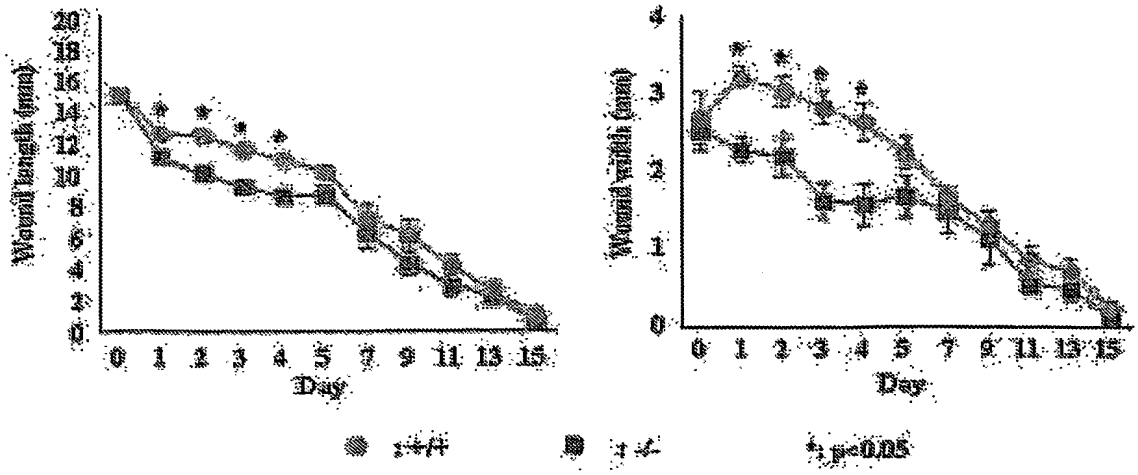
3. A method to identify molecules that comprise a region that specifically binds to AC133 comprising:
   - exposing AC133 or nucleic acids encoding AC133 to at least one molecule whose ability to suppress or prevent pathological angiogenesis is sought to be determined,
   - determining binding or hybridising of said molecule(s) to AC133 or nucleic acids encoding AC133, and
   - monitoring said pathological angiogenesis when administering said molecules as a medicament.

Abstract

The invention relates to the field of pathological angiogenesis. In particular the invention relates to the use of molecules binding to AC133 that can be used for the manufacture of a medicament to prevent angiogenesis.

5

Fig. 1: Healing of wounds in AC133 deficient mice and wild-type littermates



These data clearly indicate a role of AC 133 in pathological vasculogenesis and/or angiogenesis and implicate the use of inhibitors of AC133 in therapeutic strategies to inhibit blood vessel formation in various pathological disorders.

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<212> PRT

<213> Homo sapiens

<400> 2

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Asn Tyr Glu Leu Pro Ala Thr Asn Tyr Glu Thr Gln Asp Ser His Lys
35 40 45

Ala Gly Pro Ile Gly Ile Leu Phe Glu Leu Val His Ile Phe Leu Tyr
50 55 60

Val Val Gln Pro Arg Asp Phe Pro Glu Asp Thr Leu Arg Lys Phe Leu
65 70 75 80

Gln Lys Ala Tyr Glu Ser Lys Ile Asp Tyr Asp Lys Pro Glu Thr Val
85 90 95

Ile Leu Gly Leu Lys Ile Val Tyr Tyr Glu Ala Gly Ile Ile Leu Cys
100 105 110

Cys Val Leu Gly Leu Leu Phe Ile Ile Leu Met Pro Leu Val Gly Tyr
115 120 125

Phe Phe Cys Met Cys Arg Cys Cys Asn Lys Cys Gly Gly Glu Met His
130 135 140

Gln Arg Gln Lys Glu Asn Gly Pro Phe Leu Arg Lys Cys Phe Ala Ile
145 150 155 160

Ser Leu Leu Val Ile Cys Ile Ile Ile Ser Ile Gly Ile Phe Tyr Gly
165 170 175

Phe Val Ala Asn His Gln Val Arg Thr Arg Ile Lys Arg Ser Arg Lys
180 185 190

Leu Ala Asp Ser Asn Phe Lys Asp Leu Arg Thr Leu Leu Asn Glu Thr
195 200 205

Pro Glu Gln Ile Lys Tyr Ile Leu Ala Gln Tyr Asn Thr Thr Lys Asp
210 215 220

Lys Ala Phe Thr Asp Leu Asn Ser Ile Asn Ser Val Leu Gly Gly Gly
225 230 235 240

Ile Leu Asp Arg Leu Arg Pro Asn Ile Ile Pro Val Leu Asp Glu Ile
245 250 255

Lys Ser Met Ala Thr Ala Ile Lys Glu Thr Lys Glu Ala Leu Glu Asn
260 265 270

Met Asn Ser Thr Leu Lys Ser Leu His Gln Gln Ser Thr Gln Leu Ser
275 280 285

Ser Ser Leu Thr Ser Val Lys Thr Ser Leu Arg Ser Ser Leu Asn Asp
290 295 300

Pro Leu Cys Leu Val His Pro Ser Ser Glu Thr Cys Asn Ser Ile Arg
305 310 315 320

Leu Ser Leu Ser Gln Leu Asn Ser Asn Pro Glu Leu Arg Gln Leu Pro
325 330 335

Pro Val Asp Ala Glu Leu Asp Asn Val Asn Asn Val Leu Arg Thr Asp
340 345 350

Leu Asp Gly Leu Val Gln Gln Gly Tyr Gln Ser Leu Asn Asp Ile Pro
355 360 365

Asp Arg Val Gln Arg Gln Thr Thr Thr Val Val Ala Gly Ile Lys Arg
370 375 380

Val Leu Asn Ser Ile Gly Ser Asp Ile Asp Asn Val Thr Gln Arg Leu
385 390 395 400

Pro Ile Gln Asp Ile Leu Ser Ala Phe Ser Val Tyr Val Asn Asn Thr
405 410 415

Glu Ser Tyr Ile His Arg Asn Leu Pro Thr Leu Glu Glu Tyr Asp Ser
420 425 430

Tyr Trp Trp Leu Gly Gly Leu Val Ile Cys Ser Leu Leu Thr Leu Ile
435 440 445

Val Ile Phe Tyr Tyr Leu Gly Leu Leu Cys Gly Val Cys Gly Tyr Asp
450 455 460

Arg His Ala Thr Pro Thr Thr Arg Gly Cys Val Ser Asn Thr Gly Gly
465 470 475 480

Val Phe Leu Met Val Gly Val Gly Leu Ser Phe Leu Phe Cys Trp Ile
485 490 495

Leu Met Ile Ile Val Val Leu Thr Phe Val Phe Gly Ala Asn Val Glu
500 505 510

Lys Leu Ile Cys Glu Pro Tyr Thr Ser Lys Glu Leu Phe Arg Val Leu
515 520 525

Asp Thr Pro Tyr Leu Leu Asn Glu Asp Trp Glu Tyr Tyr Leu Ser Gly
530 535 540

Lys Leu Phe Asn Lys Ser Lys Met Lys Leu Thr Phe Glu Gln Val Tyr
545 550 555 560

Ser Asp Cys Lys Lys Asn Arg Gly Thr Tyr Gly Thr Leu His Leu Gln
565 570 575

Asn Ser Phe Asn Ile Ser Glu His Leu Asn Ile Asn Glu His Thr Gly
580 585 590

Ser Ile Ser Ser Glu Leu Glu Ser Leu Lys Val Asn Leu Asn Ile Phe
595 600 605

Leu Leu Gly Ala Ala Gly Arg Lys Asn Leu Gln Asp Phe Ala Ala Cys
610 615 620

Gly Ile Asp Arg Met Asn Tyr Asp Ser Tyr Leu Ala Gln Thr Gly Lys
625 630 635 640

Ser Pro Ala Gly Val Asn Leu Leu Ser Phe Ala Tyr Asp Leu Glu Ala
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Lys Ala Asn Ser Leu Pro Pro Gly Asn Leu Arg Asn Ser Leu Lys Arg
660 665 670

Asp Ala Gln Thr Ile Lys Thr Ile His Gln Gln Arg Val Leu Pro Ile
675 680 685

Glu Gln Ser Leu Ser Thr Leu Tyr Gln Ser Val Lys Ile Leu Gln Arg
690 695 700

Thr Gly Asn Gly Leu Leu Glu Arg Val Thr Arg Ile Leu Ala Ser Leu
705 710 715 720

Asp Phe Ala Gln Asn Phe Ile Thr Asn Asn Thr Ser Ser Val Ile Ile
725 730 735

Glu Glu Thr Lys Lys Tyr Gly Arg Thr Ile Ile Gly Tyr Phe Glu His
740 745 750

Tyr Leu Gln Trp Ile Glu Phe Ser Ile Ser Glu Lys Val Ala Ser Cys
755 760 765

Lys Pro Val Ala Thr Ala Leu Asp Thr Ala Val Asp Val Phe Leu Cys
770 775 780

Ser Tyr Ile Ile Asp Pro Leu Asn Leu Phe Trp Phe Gly Ile Gly Lys
785 790 795 800

Ala Thr Val Phe Leu Leu Pro Ala Leu Ile Phe Ala Val Lys Leu Ala
805 810 815

Lys Tyr Tyr Arg Arg Met Asp Ser Glu Asp Val Tyr Asp Asp Val Glu
820 825 830

Thr Ile Pro Met Lys Asn Met Glu Asn Gly Asn Asn Gly Tyr His Lys
835 840 845

Asp His Val Tyr Gly Ile His Asn Pro Val Met Thr Ser Pro Ser Gln
850 855 860

His
865